

Technologies for Future Measurements and Standards

- Nanobiotechnology: Nanoscale Chemical Reactions and Separations
- Protein Quantitation
- Chiral Temperature Gradient Focusing (Exploratory Research Project)

Program: Technologies for Future Measurements and Standards

Title: Nanobiotechnology: Nanoscale Chemical Reactions and Separations

Authors: L.E. Locascio, W.N. Vreeland, A. Jahn (812), and M. Gaitan (812)

Abstract: This project focuses on the development of nanoscale structures to facilitate the performance of ultra-small volume chemical reactions and separations. The work is associated with the Single Molecule Manipulation and Measurement competence program whose purpose is to study the behavior of biomolecules one molecule at a time to elucidate the differences that make them uniquely beneficial or detrimental. The nanoscale structures that we are designing are composed most often of phospholipid molecules and are self-assembled spherical or tubular structures with diameters ranging from tens to hundreds of nanometers. We have demonstrated the use of these nanometer structures for controlled chemical reactions using picoliters of reagents.

Purpose: The purpose of this work is to develop nanometer-sized structures that can ultimately be incorporated into microsystems (microfluidics and MEMS) for use in studying the behavior of very small numbers of biological molecules with fine control.

Major Accomplishments: There were two major accomplishments associated with the project during the last fiscal year:

- *Fabrication of microfluidic systems to facilitate the rapid and reproducible formation of liposomes with encapsulated fluorescent molecules*
- *Demonstration of single molecule encapsulation inside liposomes*

Due to their amphiphilic nature, when phospholipid molecules are dispersed in water they self-assemble into bilayer membranes to form structures called liposomes that are often spherical and encapsulate an aqueous internal volume. Liposomes made using bulk techniques range in size from 50 nm to 10's of micrometers encapsulating volumes that are measured in attoliters to picoliters. Water-soluble molecules can be readily incorporated into the liposomes upon formation. The ultimate goal of our work is to use liposomes as discrete packages to sequester very small amounts of reagents in order to finely control their reaction. For this purpose, two characteristics of the liposome population are critical – the liposome size and the number of encapsulated molecules contained inside each liposome. Ideally, we would like all liposomes in a given population to be identical with a diameter of approximately 100 nm and containing one encapsulated molecule. However, liposomes prepared from bulk techniques generally exhibit a very large polydispersity with either uncontrolled or unpredictable encapsulation efficiency. For example, liposomes made in our laboratory using established techniques range in size from 70 nm to 200 nm in the same preparation.

Recently, we have demonstrated the automated and controlled formation of liposomes in microfluidic systems. In this work, we hydrodynamically focus a stream of lipid tincture at a microchannel cross-junction between two aqueous buffer streams. In a typical procedure, isopropyl alcohol (IPA) containing the dissolved lipids flows through the

center inlet channel, and an aqueous phosphate buffered saline solution flows through the two side inlet channels as shown in Figure 1a. When the two liquid phases come into contact, the IPA rapidly diffuses into the aqueous phase and *vice versa*. A fluorescence image of the liposome formation process is shown in Figure 1b where the IPA solution containing lipids also contains fluorescent dye that intercalates into the formed liposome membrane. Immediately downstream of the cross intersection (to the right in the image), the fluorescent intensity of the center stream increases indicating liposome formation (the quantum efficiency of the fluorescent dye in this experiment (DiIC₁₈) increases upon incorporation into a lipid membrane). The lipids self-assemble where the concentration of alcohol and buffer mixture is at a critical condition where lipids are no longer soluble. The flow rates of the alcohol and buffer channels are adjusted to control the degree of hydrodynamic focusing influencing both the dilution rate and the shear stress at the fluid/fluid interface. We have determined that changing this parameter allows us to maintain unprecedented control over the liposome size and homogeneity. After formation, the liposomes flow downstream for collection as a tightly focused stream owing to the low Reynold's number laminar flow typical of microfluidics and the low diffusion coefficient of liposomes.

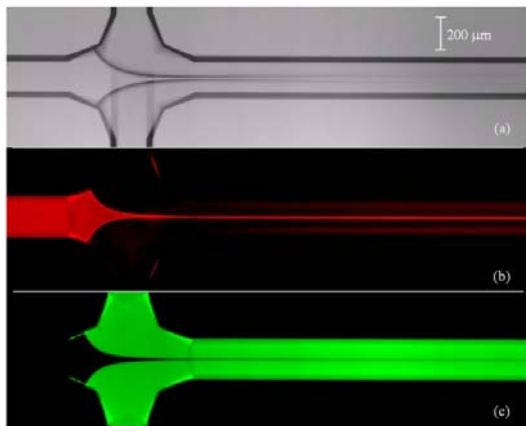


Figure 1. (a) White light image of hydrodynamic focusing of IPA by buffer streams. Fluorescence images of (b) DiIC₁₈ in IPA stream and (c) CF in buffer streams. Silicon/glass microchannels have trapezoidal cross sections with the following dimensions; depth = 40 μm, maximum width = 200 μm, minimum width = 147 μm.

We have also demonstrated encapsulation of fluorescent dye molecules into the aqueous interior of the liposomes. In these experiments, carboxyfluorescein (CF) is dissolved into the 2 buffer streams and encapsulation of the dye occurs upon liposome formation as shown in Figure 1c. The number of CF molecules encapsulated is controlled by changing the concentration of CF in the stream. Using this technique, we were able to finely tune the number of dye molecules inside the liposome population ultimately achieving single molecule encapsulation.

Impact: The characteristics of fluidic flow in a micrometer-scale channel can be used to precisely control the distribution of chemical conditions and mechanical forces so that they are constant on a length scale equivalent to that of a liposome. Hence, forming liposomes in micrometer-scale flow field results in more homogenous conditions during liposome self-assembly and resultant liposome populations that are more uniform in size, hence of low polydispersity.

Future Plans: The work is critical to our efforts in the development of new tools for observing and characterizing single molecule behavior. The characterization of single biomolecules, rather than the study of ensembles of biomolecules, is an important topic in the field of biology since it has been elucidated that the presence and behavior of the

biological outlier or the mutant version of the biomolecule can facilitate amplification of that species resulting in catastrophic consequences as highlighted in recent reports on prions. This year, we have made considerable progress toward packaging single molecules, and performing controlled reactions with a few molecules. Future work will involve further characterization of encapsulated single DNA and RNA molecules so that we can study their behavior one at a time.

Program: Technologies for Future Measurements and Standards

Title: Protein Quantitation

Authors: N. Dodder and D.M. Bunk

Abstract: To meet the need for new reference methods and materials to support clinical protein measurement, new approaches to quantitative protein mass spectrometry have been investigated. Initial studies focused on the measurement of intact proteins by MALDI mass spectrometry. Limitations of the robustness of this approach required a shift to quantitation of peptides generated from the proteolytic digestion of the analyte protein.

Purpose: Proteins are measured in the clinical setting to assist in the diagnosis of a variety of diseases including heart attacks and cancer. Currently, the majority of these tests are performed using immunoassays. Although sensitive, fast, and relatively inexpensive, the specificity, accuracy, and precision of immunoassays can be a problem and necessitates a need for method validation through protein-based reference materials and methods.

The Analytical Chemistry Division is currently developing mass spectrometric (MS) methods for protein quantitation, direct methods without the potential for bias that can be observed in an indirect method, such as immunoassay. Two methods currently under development are based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

Major Accomplishments: Initial studies explored the quantitative capabilities of MALDI-TOF mass spectrometry for the small protein human insulin. Investigations were made in sampling issues, such as the creation of homogenous sample spots, and in the automated use of the mass spectrometer, programmed to search sample spots to produce uniform sampling. Based on this research a quantitative MALDI-TOF method was developed for human insulin, using pig insulin as an internal standard. The linearity, dynamic range and error of this technique were acceptable. However, further studies, using larger proteins revealed limitations of this approach, particularly the limitations in finding internal standards that would be applicable for the measurement of large proteins.

An alternative approach is currently under investigation, using some of the methodologies developed in the growing field of proteomics. Instead of measuring intact proteins, the peptides produced from the proteolytic digests of intact proteins are measured. Using this proteomics-based approach, we are developing methods utilizing both MALDI-MS and LC/MS/MS for quantitative protein measurements.

Because proteolytic digests, particularly trypsin digests, are at the core of our proteomics-based approach, we have devoted a large effort to understanding the practical nature of

trypsin through fundamental studies of how experimental factors affect trypsin digests of analyte proteins. Guest researchers from the national metrology institutes of the United Kingdom (LGC) and Germany (PTB) have collaborated on this research effort. Successful quantitative measurements have been made on serum C-reactive protein using this approach.

Impact: As the field of proteomics matures, more protein biomarkers will be discovered and used for clinical diagnoses. New immunoassays for protein biomarkers will require validation through more metrologically-sound methods, particularly methods based on mass spectrometry. Furthermore, accurate and precise protein quantification may help answer fundamental biological questions regarding protein expression and its relation to the genome and environment. Additionally, the techniques developed for protein quantification can also be used in the areas of drug discovery and biotechnology.

Future Plans: We are developing a quantitative method for serum transferrin using a combination of ion-exchange chromatography, tryptic digestion, and MALDI-MS. A quantitative method for serum transferrin will be an important component in the on-going research at NIST to develop methods for serum iron speciation because transferrin is one of the principle serum iron transport proteins.

We will also continue the development of a proteomics-based reference method for human C-reactive protein, a clinical biomarker for mild to severe inflammation as well as for cardiovascular disease. After further refinement of the C-reactive protein reference method developed thus far, a validation study utilizing patient serum samples is planned FY05.

Program: Technologies for Future Measurements and Standards

Title: Chiral Temperature Gradient Focusing
Exploratory Research Project

Authors: D. J. Ross, K. Balss (836), K. Phinney, and W.N. Vreeland

Abstract: This project resulted from an FY04 CSTL exploratory research proposal, which was funded. Temperature gradient focusing (TGF) was demonstrated for the simultaneous concentration and separation of the enantiomers chiral molecules. The new technique was found to provide high performance in a number of areas desirable for chiral separations including rapid separation optimization and method development, facile peak order reversal (desirable for enantiomeric purity measurements), and high resolving power (comparable to capillary electrophoresis) in combination with greater than 1000-fold concentration enhancement for improved detection limits. In addition, chiral temperature gradient focusing was shown to offer a capability for real-time monitoring of the interaction of chiral analyte molecules with chiral selectors that could potentially be applied to the study of other molecular interactions.

Purpose: The goal of the project was to develop and demonstrate electrokinetic focusing methods for the high-performance separation of chiral molecules with an emphasis on molecules of biochemical and pharmaceutical interest.

Major Accomplishments: The major accomplishment of the last fiscal year (the first for this project) was to demonstrate that chiral TGF works using cyclodextrins as chiral selectors for the concentration and separation of chiral amino acids and pharmaceutical molecules. In addition, it was shown that this new technique can provide performance as good as and in some aspects superior to that provided by conventional techniques.

Specifically:

- Greater than 1000-fold concentration enhancement of chiral molecules was demonstrated.
- Chiral TGF was shown to provide resolution comparable to capillary electrophoresis (the best

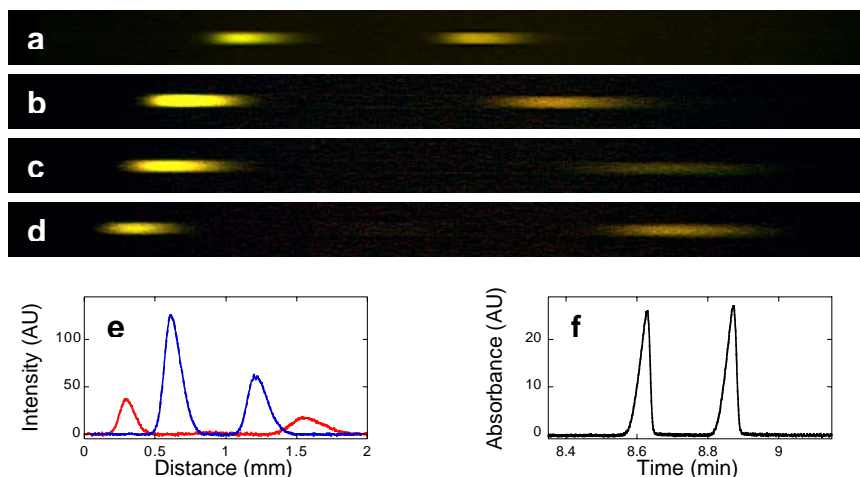


Figure 1. Optimization of chiral TGF separation of dansyl-dl-glutamic acid. (a) T1 = 13 °C, T2 = 40 °C. (b) T1 = 11 °C, T2 = 30 °C. (c) T1 = 15 °C, T2 = 30 °C. (d) T1 = 2.5 °C, T2 = 15 °C. (e) Intensity vs. distance plots for chiral TGF separations of dansyl-dl-glutamic acid; the blue curve, resolution = 2.4, is from (a); the red curve, resolution = 3.8 is from the optimized separation (d). (f) Chiral CE electropherogram of the same analyte, resolution = 4.1. CE conditions: 10 mmol/L g-CD in 15 mmol/L sodium tetraborate, 20 kV, capillary length 66 cm.

available conventional technique). This is shown in Figure 1.

- Method optimization was found to be very rapid with chiral TGF because the same focused sample could be retained and held focused in the separation channel while different temperature gradients, electric field strengths, different chiral selectors, or chiral selector concentrations were tested. In one example, chiral TGF of the drug baclofen, a γ -aminobutyric B (GABA B) receptor agonist, was tested with 4 different chiral selectors each at 8 different concentrations (32 total separation conditions) in about 2 ½ hours – with completely manual sample handling.
- Peak order reversal was also found to be very easy with chiral TGF. Simply by reversing the direction of the applied temperature gradient and the polarity of the applied voltage, the order of the focused enantiomer peaks could be reversed, facilitating the analysis of small amounts of impurity enantiomer in a nearly enantiomerically pure sample as shown in Figure 2.
- It was also found that chiral TGF of chiral drugs in urine could be successfully performed with no sample preparation. For the analysis of urine spiked with the drug baclofen, the urine was simply mixed with an equal volume of chiral TGF buffer and focused. Although there was some interference with the chiral selector due to the urine (the peaks were closer together than in a similar separation without urine), the two enantiomer peaks were still well resolved. In addition, the higher resolution of the two enantiomer peaks could be recovered by replacing the urine in the sample well with blank buffer once the drug from the urine had been focused to the desired concentration.
- Chiral TGF separations can be performed in much shorter and simpler microfluidic channels or capillaries than conventional separation techniques such as HPLC and CE. Because of this, chiral TGF, and TGF in general should be much more compatible with miniaturization and integration into microfluidic,

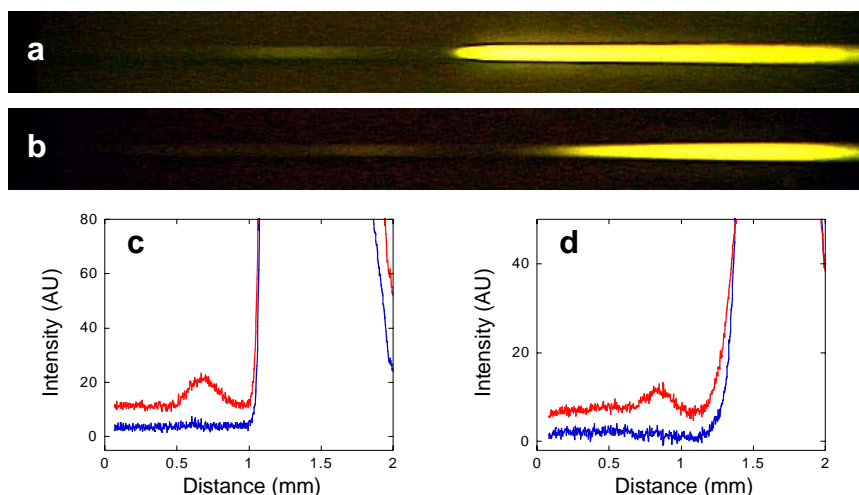


Figure 2. Trace analysis of enantiomeric purity by chiral TGF. (a) Dansyl-L-glutamic acid with 0.05 mol% dansyl-D-glu. Focusing conditions: T1 = 10 °C, T2 = 30 °C, +1000 V/cm (see Fig. 1A), 1 mol/L Tris-borate with 20 mmol/L g-CD. (b) Dansyl-D-glu with 0.1 mol% dansyl-L-glu. Focusing conditions: T1 = 40 °C, T2 = 10 °C, -1000 V/cm, 1 mol/L Tris-borate with 20 mmol/L g-CD. (c) Intensity vs. distance plots for (a, red curve) and similar results with no impurity (blue curve). (d) Intensity vs. distance plots for (b, red curve) and similar results with no impurity (blue curve). For clarity, the red curves in (c,d) have been offset.

lab-on-a-chip systems.

Future Plans: There are three directions in which this project will proceed:

- Implementation of chiral TGF with a detection mode other than fluorescence. Because most pharmaceutical molecules are not fluorescent and to avoid the necessity of chemically labeling samples, a detection mode such as UV absorbance or AC coupled conductivity will be developed for the detection and chiral analysis of dilute drugs.
- Low pH separations with TGF. Chiral separation of most pharmaceutical molecules is best performed at low pH. However, as yet, TGF has only been demonstrated with high pH (~8.5) buffers. A number of promising low pH buffers, which have the necessary temperature dependence, have been identified. They will be tested for the focusing and separation of basic drugs and other basic molecules.
- Chiral separation and detection of drugs and metabolites in body fluids for pharmacokinetic studies. Currently available separation techniques for these kinds of applications such as HPLC and capillary electrophoresis (CE) are limited either in terms of poor resolution and large sample requirements (HPLC) or poor detection limits (CE). With the combination of concentration enhancement and high resolution, chiral TGF could provide a useful alternative.